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Survival of *Listeria monocytogenes* Strain H7762 and Resistance to Simulated Gastric Fluid following Exposure to Frankfurter Exudate†

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ABSTRACT

Listeria monocytogenes strain H7762, a frankfurter isolate, was tested to determine whether it was able to survive at 4°C in frankfurter pack fluid (exudate) and to determine whether food exposure affects its acid sensitivity. Cultures were sampled and tested for acid sensitivity by challenge with simulated gastric fluid (SGF). SGF challenges performed immediately after inoculation revealed that between 20 and 26% of the cells survived the full 30 min of SGF challenge regardless of whether the cells were inoculated into brain heart infusion broth (BHI) or exudate. After 2 days of incubation, cells exposed to both exudate and BHI had significantly decreased SGF resistance; however, the cells exposed to exudate were significantly more SGF resistant than cells exposed to BHI (after 15 min of SGF treatment, 33% of the exudate-exposed cells survived and 12% of the BHI-exposed cells survived). *L. monocytogenes* exposed to exudate had greater SGF resistance at all challenge times compared with BHI-exposed cells from day 2 through day 4. From days 8 to 15, exudate-exposed cells continued to have greater SGF resistance than BHI-exposed cells up to 10 min of SGF challenge but were as sensitive as the BHI-exposed cells at 20 to 30 min of challenge. By day 25, cells exposed to exudate were significantly more sensitive to SGF challenge than BHI-exposed cells. The survivor data generated from SGF challenges were modeled by a nonlinear regression analysis to calculate the underlying distribution of SGF resistance found in the challenged populations. These analyses indicated that *L. monocytogenes* exposed to exudate at 4°C had a broader distribution of resistance to SGF compared with cells exposed to BHI at 4°C. In addition, the mean time of death during SGF treatment was greater after exposure to exudate, indicating that cells exposed to exudate were more resistant to killing by SGF. These data suggest that exposure to frankfurter exudate might render *L. monocytogenes* more able to survive the stomach environment during the initial stages of infection.

Listeria monocytogenes is a bacterium responsible for outbreak and sporadic cases of listeriosis from the consumption of contaminated foods. The bacterium has been isolated from diverse environmental sources, highlighting the organism's ability to enter the food continuum at numerous points and in a variety of ways. Although interventions during the manufacture of processed foods can effectively destroy *L. monocytogenes,* some of these foods, particularly the ready-to-eat (RTE) foods, can be contaminated postprocess, allowing *L. monocytogenes* to be present in the final product. Thus, RTE foods that are consumed without adequate reheating can be a serious public health concern. Although the incidence of listeriosis is estimated to be only five to eight cases per million in the adult population, the overall mortality rate among individuals that contract listeriosis is approximately 20 to 25% *(25).* Additionally, high-risk groups, such as pregnant women and immunocompromised individuals, are at greater risk for contracting listeriosis than the general population.

Several characteristics of *L. monocytogenes* contribute to its ability to survive and sometimes grow in foods. Most notably, the organism can tolerate relatively high levels of salt, an acidic pH, and cold temperatures *(6, 7, 15, 21).* Efforts to elucidate the mechanisms of *L. monocytogenes* survival under these stressful conditions has led to the identification and characterization of stress proteins *(1, 9, 34),* osmolyte transporters *(22, 33, 35),* degradative enzymes *(17),* and specialized sigma factors *(3, 39),* all of which play a role in stress adaptations. However, little is known about adaptations by *L. monocytogenes* that allow it to survive in food environments in which multiple stresses are often present. The exposure of *L. monocytogenes* to stressful conditions can be inferred from the sublethal injury commonly noted when *L. monocytogenes* is isolated from foods *(10, 14, 18, 24).* Because 99% of listeriosis is attributed to the consumption of contaminated foods *(25),* it is certain that in some food environments, *L. monocytogenes* can occasionally persist, survive gastric challenge, and produce an infection. Interestingly, some environmentally induced stress responses have been associated with increased virulence of *L. monocytogenes, Salmonella enterica* serovar Typhimurium, *Yersinia enterocolitica,* and other pathogens *(16, 20, 23, 31, 37, 39),* leading investigators to speculate that food environments might increase the pathogenic po-

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tential of *L. monocytogenes*. It is possible that harsh food conditions might kill the majority of *L. monocytogenes* cells but allow survival of a few cells more fit to withstand the stresses encountered during transit through the gastrointestinal tract and during the initial stages of infection.

In this study, we tested whether the frankfurter environment alters the acid sensitivity of *L. monocytogenes*. Frankfurters were chosen for this study because (i) frankfurters were identified as the vehicle for a large outbreak of listeriosis in the United States *(5)*; (ii) the U.S. Department of Agriculture Food Safety and Inspection Service (USDA/FSIS) reported 21 incidents of *Listeria*-contaminated frankfurters between 1999 and 2001, resulting in the recall of over 2.3 million pounds of frankfurters *(38)*; and (iii) it is estimated that more than 20 billion frankfurters are consumed annually in the United States *(26)*. When *L. monocytogenes* is found in finished RTE foods, the contaminating bacteria are typically introduced onto the outer surface of the food after the heating step but prior to packaging. Because bacteria contaminating the surface of frankfurters are in contact with the fluid in the package (exudate), we used frankfurter exudate as a model food system to study its effects on the acid sensitivity of *L. monocytogenes*. We exposed *L. monocytogenes* to frankfurter exudate and subsequently challenged the cells with simulated gastric fluid (SGF) as an objective measure of the cells' ability to survive the acidic stomach environment found in humans.

MATERIALS AND METHODS

Bacterial strains and growth medium. *L. monocytogenes* strain H7762, obtained from the USDA/FSIS, was originally isolated from frankfurters associated with a listeriosis outbreak. Brain heart infusion (BHI; Sigma Chemical, St. Louis, Mo.) broth and agar were used as the rich media for growth of strain H7762.

Preparation of frankfurter exudate. Fifty 1-lb (0.5-kg) packages of single-brand beef frankfurters were purchased from a local supermarket. The total volume of pack fluid (exudate) was removed from the packages by pipette, centrifuged at 5,000 rpm for 15 min to remove particulates, and then filtered through layered Whatmann (Maidstone, UK) GS/A and 0.45-μm Nalgene (Nalge, Rochester, N.Y.) filters. The filtrate was given a final filtration through a 0.2-μm Nalgene filter and stored at 4°C.

Preparation of SGF. The SGF used in this study was prepared essentially according to Beumer et al. *(4)*. The porcine bile used was Hog Bile Extract (ICN Biochemicals, Aurora, Ohio). HCl (1 N) was added to adjust the SGF to pH 1.5 as measured by a Corning pH meter 340 (Corning, N.Y.).

Survival of H7762 in SGF. Two single-colony isolates of H7762 were used to inoculate two 10-ml tubes of BHI broth. After shaking at 30°C overnight, the cultures were centrifuged at 5,000 rpm for 15 min to pellet the cells. One pellet was resuspended in BHI preequilibrated to 4°C to achieve a final optical density at 600 nm (OD_{600}) of approximately 1.5. The second pellet was treated similarly except the cells were resuspended in exudate preequilibrated to 4°C to achieve a final OD_{600} of approximately 1.5. The BHI and exudate suspensions were diluted 1:10 into duplicate 15-ml aliquots of either BHI or exudate preequilibrated to 4°C to achieve a final concentration of approximately 1×10^8 CFU/ml. The 15-ml BHI and exudate cultures were shaken (100 rpm) at 4°C for 25 days. The cultures were sampled on days 0 (immediately after inoculation), 2, 4, 6, 8, 10, 15, and 25 and plated onto BHI agar and BHI plus 5% NaCl agar to determine cell counts (CFU/ml) and to detect injury. In parallel, 500 μl of the culture was added to 4.5 ml SGF preheated to 37°C. All SGF challenges were conducted at 37°C. At 5-min intervals extending to 30 min, 400-μl aliquots were removed and plated on BHI agar to determine the number of survivors. The percent survival data was obtained by combining values for duplicate cultures, and the complete experiment was replicated to confirm results.

Modeling and statistical analyses. Analysis of the SGF challenge data was performed by the Mixed Procedure of SAS incorporating repeated measures (SAS Institute Inc., Cary, N.C.). To determine the underlying distributions of sensitivity to SGF, the equation $S(t) = \exp(-bt^n)$ was used to fit the survival data as described by Peleg and Cole *(27)*. The values obtained for b and n were then used to calculate the distribution's mode, mean, variance, and coefficient of skewness. These values were used in SAS/STAT to generate a plot of the distributions of sensitivity.

RESULTS

Survival of strain H7762 in frankfurter exudate. Immediately after inoculating *L. monocytogenes* into exudate or BHI, samples were removed and plated onto BHI agar to determine the total number of viable cells. Initial cell counts were approximately 1×10^8 CFU/ml. A high initial starting density was used because preliminary experiments indicated that a low inoculum would not provide a sufficient number of cells to assess the effect of the SGF challenge (data not shown). On sampling days, the exudate and BHI cultures were plated onto BHI agar and BHI agar plus 5% NaCl to determine the numbers of uninjured and injured bacteria; however, there were no significant differences in the bacterial counts (data not shown). The total bacterial numbers determined from direct plating onto BHI agar were used to track overall survival of the cultures throughout the incubation period. Both the exudate and BHI cultures decreased less than 0.5 log CFU/ml after 2 days of incubation at 4°C. The viable cell count remained fairly constant in the BHI cultures from day 0 to day 6; however, from day 6 to day 25 there was a small increase in cell number that resulted in an increase of approximately 0.5 log CFU/ml. In contrast, the viable cell count of the exudate cultures slowly decreased 0.5 log CFU/ml between day 2 and day 25, indicating that the exudate environment had a slight negative effect on cell viability.

Sensitivity to simulated gastric fluid following preincubation at 4°C. The SGF treatment time had a significant effect on all days, which was reflected by a reduction in bacterial viability in response to SGF. On day 0, there was not a significant difference in SGF resistance between cells in BHI or exudate. On inoculation into exudate or BHI (Fig. 1a), approximately 20 to 26% of the cells survived the 30-min SGF treatment regardless of the pre-SGF challenge media. The SGF resistance of cells exposed to either BHI or exudate decreased by day 2 (Fig. 1b). There was also a significant effect due to the incubation media. Specifically, the level of resistance was significantly greater for

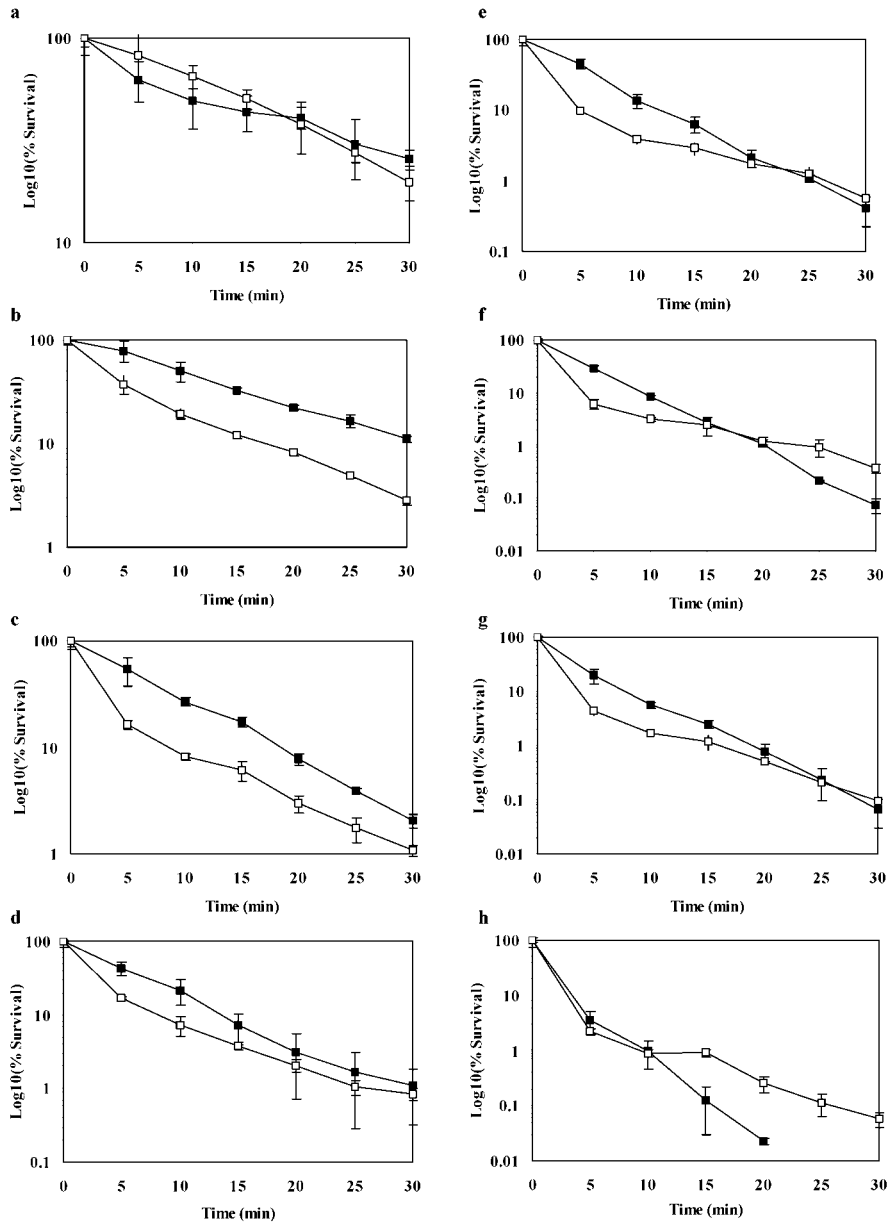

FIGURE 1. *Survival of* L. monocytogenes *H7762 in simulated gastric fluid following preincubation at 4°C in frankfurter exudate or BHI broth. Percent survival was calculated after challenge by SGF (a) immediately after inoculation into BHI (□) or exudate (■) and at days 2 (b), 4 (c), 6 (d), 8 (e), 10 (f), 15 (g), and 25 (h) post-inoculation.*

exudate-exposed cells compared with BHI-exposed cells at all SGF sampling times on day 2. At 15 min of SGF treatment, 33% of the exudate-exposed cells survived compared with only 12% of the BHI-exposed cells. Similarly, at 30 min of SGF treatment, 11% of the exudate-exposed cells survived compared with only 2% of the BHI-exposed cells. The statistical analysis also indicated that the difference in the mean number of survivors was more disparate with increasing SGF time. On day 4 of sampling, the BHI-exposed cells were still significantly more sensitive to SGF challenge than the exudate-exposed cells (Fig. 1c). This was similar to the day 2 results; however, there was no evidence of an interaction between media and SGF challenge time, and the magnitude difference in the mean number of survivors was similar at the SGF challenge times. On days 6 and 8, the exudate-exposed cells were more SGF resistant than the BHI-exposed cells for the first 15 min of SGF challenge (Fig. 1d and 1e); however, the difference did not reach statistical significance on day 6 because of a large variance for the day 6 samples. By days 10 to 15, the exudate-exposed cells remained significantly more resistant than the BHI-exposed cells during the first 10 min of SGF treatment, but by 15 min, the sensitivities of both types were similar (Fig. 1f and 1g). On day 25, both exudate-exposed and BHI-exposed cells were highly and equally sensitive to SGF challenge during the first 10 min of SGF treatment, but the BHI-exposed cells were more resistant at the 15- and 20-min sampling times compared with the exudate-exposed cells (Fig. 1h). The number of survivors among the exudate-exposed cells had decreased to below detectable levels (<2 log CFU/ml) by the 25-min SGF challenge sampling time.

To further explore the changes in SGF sensitivity over the 25-day experiment, the percent survival data for the 5- and 30-min SGF challenge time points were graphed relative to day of sampling (Fig. 2a and 2b). The most dramatic differences in SGF sensitivity between the exudate- and BHI-exposed cells occurred in the first 5 min of SGF chal-

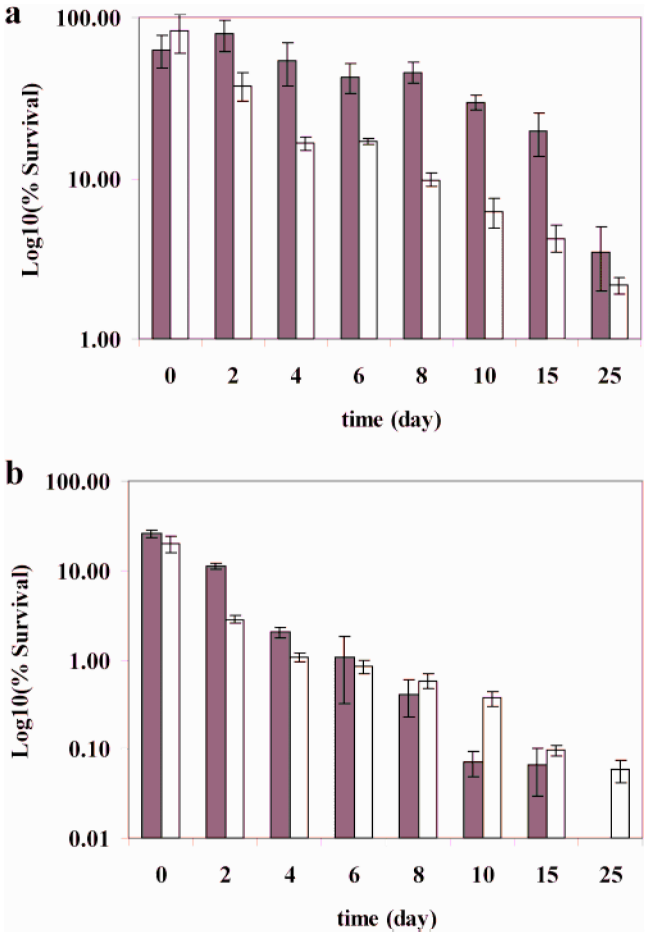

FIGURE 2. *The SGF sensitivity of* L. monocytogenes *H7762 over a 25-day, 4°C incubation in exudate or BHI broth. Percent survival data after 5-min (a) and 30-min (b) challenges with SGF were combined from days 0, 2, 4, 6, 8, 10, 15, and 25. The exudate and BHI preincubation conditions are represented by the black and white columns, respectively.*

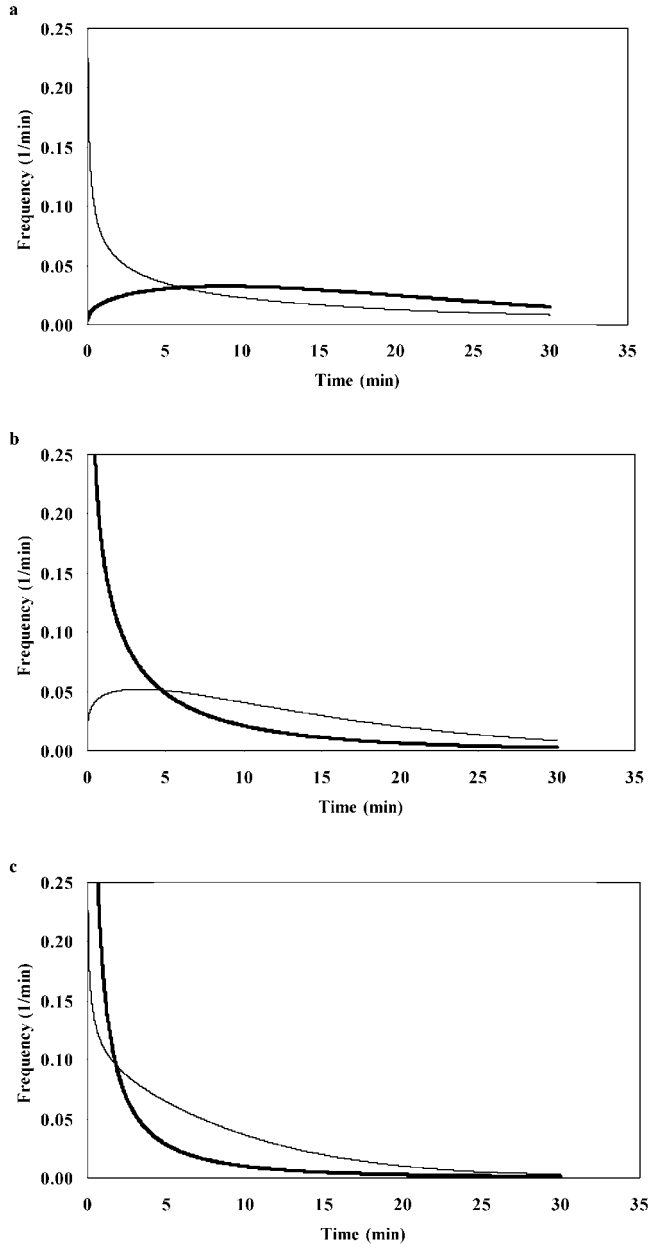

FIGURE 3. *The distribution of sensitivity to SGF for* L. monocytogenes *H7762 preincubated in exudate or BHI broth. The distribution of sensitivity to SGF is plotted for days 0, 2, and 4 (plots a, b, and c, respectively) for strain H7762 preincubated in BHI (▬) or exudate (——). The frequency is expressed on the* y *axis as a measure of the lethal events within the population for any given time during SGF treatment.*

lenge, since exudate-exposed cells were significantly more resistant to SGF than BHI-exposed cells on all sampling days except days 0 and 25 ($P < 0.02$; Fig. 2a). Conversely, SGF challenge for 30 min resulted in less dramatic differences between the exudate- and BHI-exposed cells because only days 2 and 4 show a significantly more SGF-resistant exudate population when compared with the BHI-exposed cells (Fig. 2b). In addition, the BHI-exposed cells were more SGF-resistant than the exudate-exposed cells after 30 min of SGF treatment on days 10 and 25.

The pH values of the BHI and exudate cultures were tested at several points throughout the incubation period, because the differences in SGF resistance observed between the BHI- and exudate-exposed cells might be partially a result of the exudate environment inducing a pH-dependent acid tolerance response. The pH of the exudate cultures ranged from 5.9 to 6.0, whereas the pH of the BHI cultures ranged from 7.1 to 7.3, indicating that the exudate environment was slightly acidic. *L. monocytogenes* was unable to significantly alter the pH of either the exudate or BHI cultures because the pH remained within the starting pH range throughout the course of experimentation. Even though the pH readings remained constant, the SGF sensitivity of both cultures changed over time as indicated previously.

Analysis of SGF challenge data by nonlinear regression and the Weibull distribution. The survivor data for SGF challenges performed on days 0 to 25 were fit to a model by nonlinear regression assuming a Weibull distribution as described by Peleg and Cole *(27).* Rather than depicting survival, Figure 3 shows the distribution of resistance to SGF within the *L. monocytogenes* population for the exudate and BHI exposure conditions on days 0, 2, and 4. The curves in Figure 3 graphically depict the distribution

TABLE 1. *Mean time of death for* L. monocytogenes *H7762 after challenge with SGF following 4°C incubation in exudate (EX) or brain-heart infusion broth (BHI)*

Day	Mean time of death (min) EX (±SD)	BHI (±SD)	*F* statistic[a]	*P*[b]
0	27.28 ± 2.74	19.13 ± 2.57	54.87	<0.0001
2	13.88 ± 0.01	6.54 ± 0.12	44.48	<0.0001
4	7.97 ± 0.78	3.33 ± 0.23	17.73	0.0009
6	6.07 ± 1.26	2.92 ± 0.23	8.21	0.0128
8	5.27 ± 0.47	1.99 ± 0.19	8.86	0.0103
10	4.25 ± 0.32	1.53 ± 0.22	6.10	0.0274
15	3.46 ± 0.25	1.18 ± 0.27	4.28	0.0580

[a] *F* statistic was obtained from a planned comparison analyzed with the SAS Mixed procedure and a repeated measures ANOVA.

[b] Probabilities were determined using the *F* statistic and a pooled estimate of error.

of resistance and are indicative of cell variability in response to the SGF challenge. Figure 3a shows the distribution of resistance in the day 0 populations as derived from modeling of the survival data that was plotted as percent survival in Figure 1a. The distribution curves indicate that the majority of the BHI-exposed cells were slightly more resistant to SGF than were the exudate-exposed cells. In comparison to day 0, there was a change in the distribution of resistance for both the exudate-exposed and BHI-exposed cells on day 2. The BHI-exposed cells had become much less resistant to SGF challenge, whereas the exudate-exposed cells had become more resistant to SGF challenge (Fig. 3b). The distribution of resistance within exudate-exposed cell cultures was clearly broader compared with the distribution of resistance within BHI-exposed cell cultures. This is corroborated in the inactivation curve in Figure 1b and the corresponding analysis of statistical difference of mean time of death (Table 1). The distribution of resistance for sampling day 4 is shown in Figure 3c and indicates increasingly uniform and less resistant populations for both the exudate-exposed and BHI-exposed cells. The distribution plots for both the exudate- and BHI-exposed cells became increasingly narrow for subsequent sampling days 6, 8, 10, 15, and 25, suggesting a population of cells that are more uniform in their resistance to SGF challenge (data not shown).

The mean sensitivity values, obtained by modeling the data, are indicative of the challenge time when the typical cell within the population was killed by the SGF treatment. This average time of death measurement is a useful indicator of difference in SGF sensitivity between the exudate-exposed and BHI-exposed cells. Table 1 shows the results of the analysis of variance (ANOVA) obtained by analyzing the mean time of death values generated from SGF challenges of BHI-exposed and exudate-exposed cells. The ANOVA was performed with the Mixed procedure of SAS, incorporating repeated measures. As expected from the percent survival data, the mean death time decreased for both conditions from days 0 to 15, indicating an increasing sensitivity for both exudate-exposed and BHI-exposed cells as a function of incubation time. The mean death times for the exudate-exposed cells were significantly higher than those for the BHI-exposed cells on all days except day 15, when the cells were not significantly different in their sensitivity to SGF. An ANOVA of the average variance in the distributions indicated that the variance on day 0 and day 2 in the BHI-exposed populations (130.47 ± 1.84 and 65.25 ± 0.39, respectively) was significantly less than the variation in the exudate-exposed populations (210.41 ± 45.73 and 123.29 ± 27.38, respectively); $P = 0.0001$ and $P = 0.0018$, respectively. After day 2, the variation was not significantly different between the different exposure conditions. This type of modeling analysis illustrates differences in SGF sensitivity that are not readily apparent when examining only the percent survival data shown in Figure 1e through 1g.

DISCUSSION

Several studies have been conducted to identify *L. monocytogenes* stress response proteins and to identify the roles these proteins might play in virulence *(16, 17, 30, 34, 35, 39).* In addition, some researchers have focused on how food environments might induce adaptations that affect the ability of *L. monocytogenes* to survive in foods *(14, 18, 24).* However, few studies have addressed the issue of whether adaptations in food environments can increase the virulence potential of *L. monocytogenes* cells that survive in foods. In this study, we examined whether frankfurter exudate affects *L. monocytogenes* survivability at 4°C and the subsequent ability of the bacteria to withstand SGF challenge.

When *L. monocytogenes* was incubated at 4°C in exudate, approximately 27% of the cells were able to survive a 25-day exposure to the slightly acidic and high-osmolarity exudate environment. Furthermore, testing for injury by plating onto BHI and BHI plus 5% NaCl did not indicate *L. monocytogenes* was injured following cold incubation in exudate, in contrast to studies that have indicated that long-term chilled storage can reduce the ability of *L. monocytogenes* to grow in a high-salt medium *(13).* Although it was surprising that low temperature and exudate components such as salt, acids, and phenolic compounds did not result in measurable cell injury, it is possible that the cells were adapted to salt stress by the high osmolarity of the exudate and that injury could not be adequately assessed by plating onto BHI agar containing 5% NaCl. Although the composition of the frankfurter exudate was not analyzed, frankfurter formulations generally average 2.3% salt by weight. Given the reduction in water that occurs by cooking, it is likely that the salt content of the frankfurter exudate used in this study is comparable to BHI with 5% added NaCl. An alternative might be that *L. monocytogenes* strain H7762 has a selective advantage for survival in the frankfurter environment, given that the strain was isolated from frankfurters. If *L. monocytogenes* strain H7762 is more able to withstand a frankfurter environment, it might have an increased ability to take up or use compatible solutes such as carnitine and acetyl carnitine, common con-

stituents of meat products. Uptake of these and other protective solutes have a demonstrated beneficial effect for survival and growth under conditions such as low temperature in foods *(2, 36)*. Furthermore, *L. monocytogenes* strain H7762 was isolated from frankfurters linked to an outbreak of listeriosis, which could suggest that the strain has an enhanced ability to adapt to the food environment. Similar strain adaptations have been implicated in a study showing that clinical strains were better able to utilize carnitine as a protective osmolyte when compared with meat isolates *(11)*.

The survival of *L. monocytogenes* was assessed in SGF following BHI exposure or frankfurter exudate exposure at 4°C in an attempt to quantify changes in virulence potential resulting from exposure to a food. The data were analyzed by percent survival and nonlinear regression and then modeled assuming a Weibull distribution in an effort to describe inactivation on the basis of the underlying distribution of SGF resistance in the population. This type of analysis has been used to analyze both linear and nonlinear microbial survival data *(27–29)*. In general, the percent survival and nonlinear regression analyses generated similar conclusions. Both analyses indicated that BHI-exposed and exudate-exposed cells survived SGF fairly well on day 0, but the exudate-exposed cells were more SGF resistant than the BHI-exposed cells on later days of sampling. This trend continued through day 10 of sampling. The mean death times generated from the modeled data revealed a significantly less resistant population of BHI-exposed cells from days 0 to 10 in contrast to the percent survival data, which showed no significant differences in survival rates between BHI- and exudate-exposed cells. Although the 30-min day 0 SGF challenge resulted in very little mortality for both the exudate-exposed and BHI-exposed cells, there are differences evident in the Figure 1a inactivation curves. The modeled data reflect these differences as the distribution of resistance in these populations. Another important conclusion derived from the modeled data is that, compared with BHI-exposed cells, the population of exudate-exposed cells was significantly more variable in their response to SGF challenge on day 0 and day 2. The distribution of SGF resistance within a group of cells contaminating a food might be an important consideration for food safety because it could be indicative of the population's ability to survive gastric challenge and cause disease. If a number of *L. monocytogenes* cells present in food remain relatively resistant to gastric fluid, these cells could significantly reduce the required infective dose. In support of this theory is a study demonstrating that acid-adapted *L. monocytogenes* were more infective in a mouse intragastric model, suggesting that acid adaptation is advantageous to surviving gastric fluid in vivo *(32)*.

Temperature, phase of growth, starvation, and the presence of osmolytes are all factors that independently have been shown to affect the ability of *L. monocytogenes* to survive, mount stress responses, and cause infection *(2, 8, 12, 19)*. The effects that these and other combined stresses have on *L. monocytogenes* need to be considered in foods in which such stresses are present. Other conditions associated with RTE foods that could influence *L. monocytogenes* physiology and pathogenesis (pH, salt, competitive flora) and differences among brands and types of food products should also be considered. In our tests, the pH of the exudate cultures was slightly acidic, indicating that the exudate environment might produce a pH-dependent acid tolerance response, which could explain some of the differences observed during SGF challenge. The *L. monocytogenes* acid tolerance response has been shown to increase resistance to acid challenge, but whether this increased acid resistance increases virulence is not known *(8, 16)*. Because neither the exudate nor BHI cultures had a change in pH during the time frame of the study, the change in the level of SGF sensitivity could be a result of long-term adaptations to pH, some factor other than pH, or most likely, a complex interaction of stresses. Along with pH, the other stressful conditions mentioned would also be expected to differ in various frankfurter formulations. Unique formulations would likely result in different *L. monocytogenes* responses but were beyond the scope of our initial study to test. In the future, experiments focusing on *L. monocytogenes* strain differences, the environmental history of the cells, and the variable aspects of foods will be useful in elucidating the *L. monocytogenes* responses to food environments. Determining these responses will allow better predictions to be made regarding how such responses might influence the ability of the organism to cause infection.

The results of this study indicate that food environments can increase the ability of *L. monocytogenes* to survive a model gastric challenge and imply that the virulence potential of the organism might increase on contact with food. Determining the factors that influence *L. monocytogenes* virulence provides direction for establishing food processing interventions that reduce the ability of *L. monocytogenes* to cause infection.

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